INHIBITION OF LIPID SYNTHESIS IN *BACILLUS AMYLOLIQUEFACIENS* BY INHIBITORS OF PROTEIN SYNTHESIS

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SUMMARY: Inhibitors of protein synthesis reduce the level of lipid production in *Bacillus amyloliquefaciens*, both in growing cells and in non-dividing washed-cell suspensions. This effect is primarily on phospholipids rather than acetone-soluble lipids. Although the cells contain five major phospholipids the washed-cell supernatants contain only one phospholipid species whose accumulation is sensitive to chloramphenicol but insensitive to rifampicin.

INTRODUCTION

It has been reported that inhibition of protein synthesis in *Bacillus subtilis* 168 by chloramphenicol resulted in a reduction in the rate of lipid synthesis.[1,2] On the other hand, it has also been reported that chloramphenicol did not affect lipid synthesis in *Mycoplasma laidlawii* or *Escherichia coli*.[3,4] In this paper it is shown that in *Bacillus amyloliquefaciens* several inhibitors of protein synthesis have marked effects on lipid synthesis.

MATERIALS AND METHODS

B. amyloliquefaciens was grown at 30°C from a spore inoculum in a salts-maltose-Casamino acids medium.[5] Washed-cell experiments were carried out as described previously.[6] Protein and RNA synthesis were measured by the incorporation of L-[^{14}C]phenylalanine and [^{14}C]-uracil as described previously.[6] Lipid synthesis was measured by the incorporation of [^{14}C]-2-glycerol into TCA precipitable material. It has been shown that essentially all the incorporated glycerol is recoverable in the extracted lipid fraction.[7]

Lipids were extracted by the method of Houtsmuller and van Deenan,[8] except that the buffer was citrate-phosphate (pH 6.5). Phospholipids were separated by thin layer chromatography on Eastman 6061 silica gel chromatogram sheets using solvent systems 1, $CHCl_3$:CH_3OH:H_2O (65:25:4 v/v) and 2, $CHCl_3$:CH_3OH:7MNH_4OH (60:35:5 v/v).[9] Phospholipids were detected by iodine vapour and the molybdate method of Vaskovsky and Kostetsky.[10]

Radiochemicals were from Schwarz-Mann, apart from [^{14}C]-2-glycerol which was from New England Nuclear.

RESULTS AND DISCUSSION

B. amyloliquefaciens was grown for 24 hours in the salts-maltose-Casamino acids medium and the effect of chloramphenicol (100 µg/ml), added to the cultures, on protein and lipid synthesis examined. It was found that at this concentration chloramphenicol inhibited protein synthesis by 98-99% and that of lipid by 63%. Similar results were obtained with pactamycin (1.0 µg/ml) and tetracycline (1.0 µg/ml) which inhibited protein synthesis by 98% and 85% and lipid synthesis by 57% and 44% respectively (Fig. 1a,1b). The incorporation into lipid was linear from zero time and the inhibition instantaneous.

In order to check that these effects were not the result of general toxic effects of antibiotics on cells, the effect of these drugs on RNA synthesis was examined. Cells were cultured in the usual manner and after 24 hours [^{14}C]-uracil (0.2 µC.ml) and unlabelled uracil (4.0 µg/ml) were added to the culture. There was a marked stimulation of RNA synthesis in the drug-treated cultures (Fig. 1c).

Similar results were observed in washed-cell experiments, where growth was restricted by nutrient limitation.

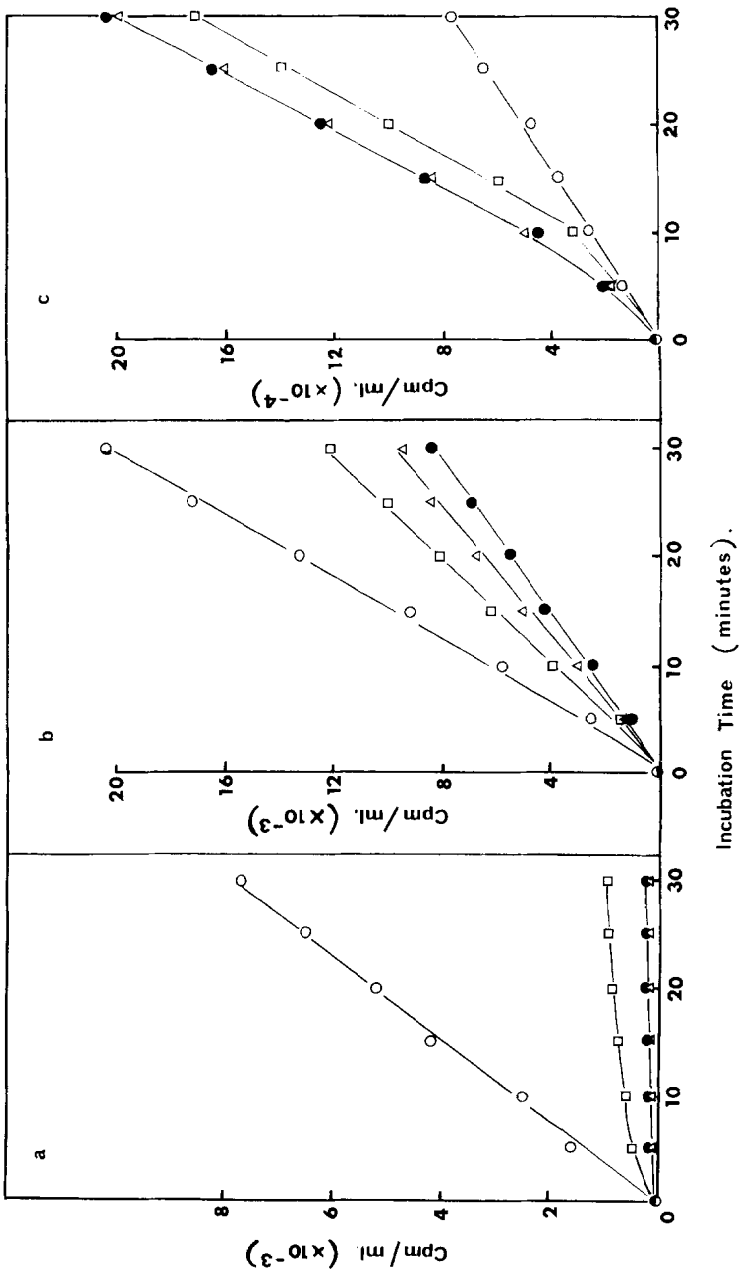
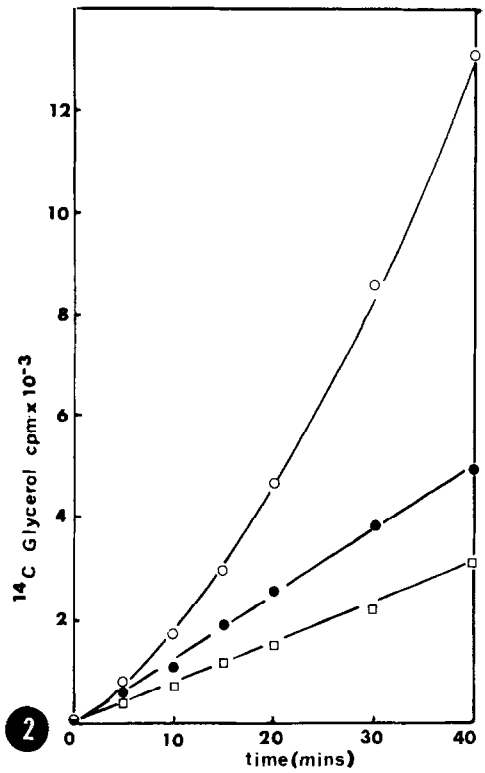


FIG. 1. The effect of inhibitors of protein synthesis on protein, lipid and RNA synthesis in growing cells of *B. amyloliquefaciens*. Twenty-four hour cells in the Casamino acid growth medium were either untreated (○) or exposed to chloramphenicol (100 µg/ml) (●), pactamycin (1.0 µg/ml) (△), tetraycline (1.0 µg/ml) (□) and the incorporation of [^{14}C]-L-phenylalanine (Fig. 1a), [^{14}C]-2-glycerol (Fig. 1b) and [^{14}C]uracil (Fig. 1c) into TCA precipitable material compared to untreated cells. Samples (0.1 ml) were taken at intervals and treated as described previously.



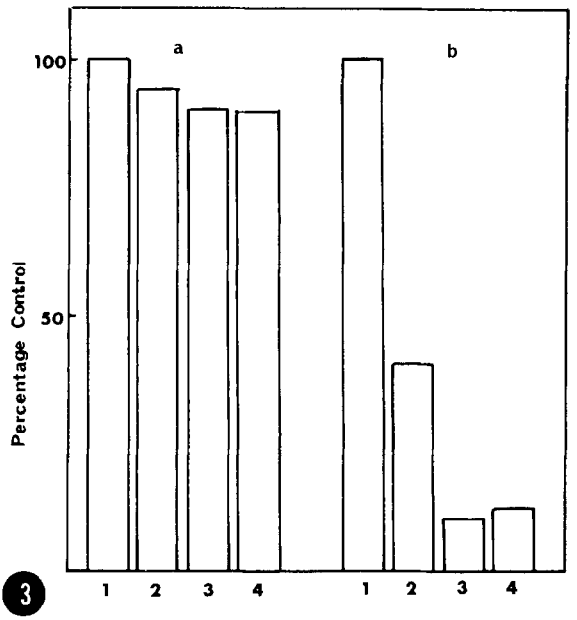


FIG. 2. The effect of rifampicin and chloramphenicol on lipid synthesis in washed-cell suspensions in *B. amyloliquefaciens*. Washed-cell suspensions were incubated with [^{14}C]-2-glycerol (0.25 µC/ml) and unlabelled glycerol (200 µg/ml). At zero time chloramphenicol (10 µg/ml) (□) or rifampicin (0.5 µg/ml) (●) was added to a sample of cells; control cells (O) were incubated without drugs. Samples (0.1 ml) were taken at intervals into 5% TCA containing 0.05% unlabelled glycerol.

FIG. 3. The effect of chloramphenicol and rifampicin in cellular lipids. Cells in washed-cell suspension medium were treated with chloramphenicol (2 µg and 10 µg/ml) and rifampicin (0.5 µg/ml) at zero time and the lipids extracted after 90 minutes incubation at 30°C.

FIG. 3a. Effect on acetone-soluble lipids 1, control; 2, chloramphenicol (2 µg/ml); 3, chloramphenicol (10 µg/ml); 4, rifampicin (0.5 µg/ml). The 100% value was 5188 µg.

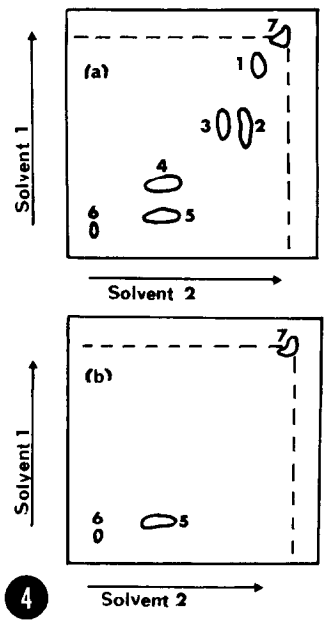
FIG. 3b. Effect on phospholipids. 1, control; 2, chloramphenicol (2 µg/ml); 3, chloramphenicol (10 µg/ml); 4, rifampicin (0.5 µg/ml). The 100% value was 1405 µg. The values are averaged from two separate gravimetric estimations.

Under these conditions chloramphenicol (10 µg/ml) and rifampicin (0.5 µg/ml), (concentrations which inhibited general protein synthesis by 95-98%) inhibited lipid synthesis by 74% and 67%

respectively (Fig. 2). Additional experiments have shown that pactamycin (1.0 µg/ml) and fusidic acid (20 µg/ml) inhibited lipid synthesis by 74% and 70% respectively. It should be pointed out that growing cells seem to be more resistant to chloramphenicol than non-growing cells. Separate experiments have shown that the effects of chloramphenicol and pactamycin are completely reversible by collecting and washing the cells on millipore filters. Cells from which drugs have been removed in this manner incorporate L-[^{14}C]phenylalanine and synthesise protease at rates identical with control cells not exposed to antibiotics.[11] These results strongly suggest that the effect of these inhibitors on lipid formation was not the result of a general toxic effect.

In order to test whether these drugs were affecting acetone-soluble lipids or phospholipids, or both, the following experiment was carried out. Washed-cells (40 ml) were incubated with shaking for 90 min in washed-cell suspension medium containing 2 µg/ml chloramphenicol, 10 µg/ml chloramphenicol, 0.5 µg/ml rifampicin and a control with no drugs added. After 90 min the cells were extracted for acetone-soluble lipids and phospholipids by the method of Houtsmuller and van Deenan.[8] When the acetone-soluble lipids were examined it was found that addition of drugs had little or no effect ($\pm$ 10% of control) (Fig. 3a) on the level of these lipids in the cells. On the other hand, there was a dramatic reduction in the level of phospholipids in the cells when compared with the untreated control cells (Fig. 3b).

This result shows that when lipid synthesis is affected by inhibitors of protein synthesis the effect is primarily on the phospholipid fraction. When the phospholipids extracted from cells were examined by thin layer chromatography on silica gel plates, five major phospholipid spots were observed when stained



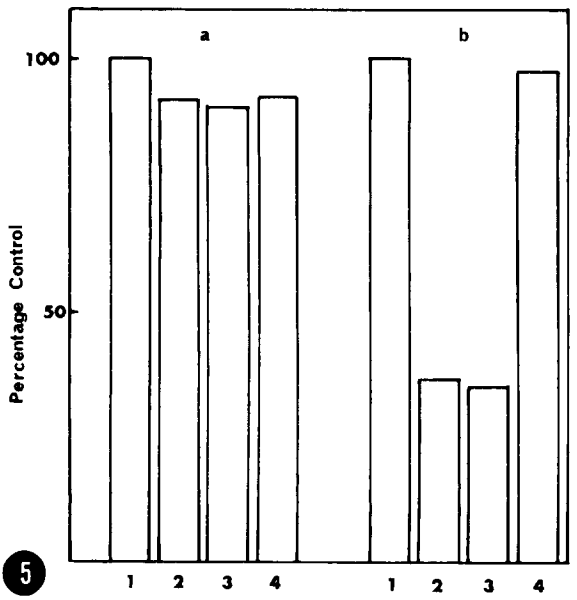


FIG. 4. Phospholipids extracted from cells of *B. amyloliquefaciens*. Species 1-7 stained with I vapour, 1-6 with molybdate reagent and 3 and 4 gave a positive reaction with ninhydrin (1% (w/v) in acetone). Species 6 is unmigrated phospholipid while species 7 are contaminating acetone-soluble lipids.

FIG. 4a. Phospholipid extracted from the washed-cell supernatant after 90 minutes incubation at 30°C. Species 5 co-migrates with the species 5 extracted from washed-cells and does not stain with ninhydrin.

FIG. 5. The effect of chloramphenicol and rifampicin on lipids in washed-cell supernatants. The experiment was carried out as described in Fig. 4 except that the supernatant fraction was extracted and analysed.

FIG. 5a. Effect on acetone-soluble lipids. 1, control; 2, chloramphenicol (2 µg/ml); 3, chloramphenicol (10 µg/ml); 4, rifampicin (0.5 µg/ml). The 100% value was 4693 µg.

FIG. 5b. Effect on supernatant phospholipid. 1, control; 2, chloramphenicol (2 µg/ml); 3, chloramphenicol (10 µg/ml); 4, rifampicin (0.5 µg/ml). The 100% value was 445 µg.

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with iodine vapour. All of these spots were stained with the molybdate reagent described by Vaskovsky and Kostetsky[10] and two of the phospholipids stained positively with ninhydrin (1% w/v in acetone) (Fig. 4). The pattern and staining of the phospholipids were almost identical to that of Oo and Lee.[9] When phospho-

lipid samples extracted from cells treated with chloramphenicol (10 µg/ml) or rifampicin (0.5 µg/ml) were examined by thin layer chromatography the results indicated that there was a general reduction of all phospholipids rather than a selective reduction.

In order to eliminate the possibility that the phospholipids were being excreted into the supernatant rather than remaining associated with the cell, the culture supernatants were examined. The experimental procedure was exactly as described for the last experiment. At the end of the incubation period the washed-cell supernatants were examined for acetone-soluble lipids and phospholipids. It was found that the supernatants contained quite large quantities of acetone-soluble lipid (as evidenced by the fact that cell cultures of this organism when centrifuged often show a lipid pellicle). When these were examined by thin layer chromatography, all those species in the supernatant were also found in the cells. When the phospholipids were examined, however, it was found that only a single phospholipid species was present in the supernatant (Fig. 4b). When the effect of chloramphenicol (2 µg/ml and 10 µg/ml) and rifampicin (0.5 µg/ml) on the levels of phospholipid in the supernatant was examined it was surprisingly discovered that whereas chloramphenicol gave a reduction in the amount of the supernatant phospholipid, rifampicin did not markedly affect the level of this species (Fig. 5). Separate experiments were carried out in which the medium itself was extracted: it was found to contain 518 µg acetone-soluble lipids/40 ml but no detectable phospholipids. It should be mentioned that the supernatant phospholipid species co-chromatographed with spot 5 of the cell phospholipids (cf. Fig. 4a and 4b).

Since in the presence of rifampicin it is known that synthesis of extracellular protease will continue for 80 minutes,

α-amylase for 60 minutes and ribonuclease for 40 minutes,[6 12] it is unlikely that normal phospholipid synthesis is required for extracellular enzyme secretion. This does not eliminate the possibility, however, of special sites in the membrane which may be linked to extracellular enzyme secretion, synthesis of whose lipid components is insensitive to rifampicin. Although the synthesis of the phospholipid in the supernatant is chloramphenicol sensitive and rifampicin insensitive, just as extracellular enzymes are in this organism, any possible role of this species in extracellular enzyme formation must remain speculative at the present time.

The results presented here showing the effect of inhibitors of protein synthesis on phospholipid synthesis could be a reflection of the decay of proteins involved in phospholipid synthesis. If such were the case the rate of decay of these enzymes would have to be almost immediate to account for the results in Fig. 1b. An alternative and more likely explanation is contained in the idea advanced by Mindich[1] that incorporation of proteins into the membrane loosely controls lipid incorporation.

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